Comparative analysis of the sequence of the genomes *'Ca.* Phytoplasma' species

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Bacteria of the genus '*Candidatus* Phytoplasma' are uncultivated intracellular plant pathogens transmitted by phloem-feeding insects. They have small genomes lacking genes for essential metabolites, which they acquire from either plant or insect hosts. As a result of the complete sequencing of the genomes of six phytoplasma strains, a great success has been achieved in understanding the effects of phytoplasma on plants and insects at the molecular level. Comparative analyses of the six sequenced phytoplasma genomes unveiled diversity in genome size, composition, metabolic pathways and the number of repeats. It is assumed that rapid genome evolution of phytoplasmas is attributed to the consequence of their life cycle. Both organisms are required for the continuous life and distribution cycle of phytoplasmas among plants and insects in nature. This requires adaptation to a broad range of environments, including the phloem of their plant hosts and the gut lumen, haemolymph, saliva and endocellular niches in various organs of their insect hosts. Obtained data on phytoplasma genome sequence led to the identification of a number of potential virulence proteins, some of which were functionally characterized and their role in insect invasion of plants was confirmed. These include proteins on the phytoplasma cell surface involved in binding of insect gut microfilaments and secreted proteins that target plant cell nuclei. The large repeats are present in the majority of phytoplasma genomes, which are expressed in phytoplasma virulence.

Keywords*: Phytoplasma DNA, complete and draft genome sequences, potencial mobile units*

INTRODUCTION

Phytoplasmas are plant-pathogenic bacteria that can infect over 700 plant species, transmitted by insects, causing devastating crop damage worldwide. Infected plants show a wide range of symptoms, e.g. dwarfism, witches'broom, yellowing, purple top, and phyllody (Hogenhout et al., 2008). It is known that phytoplasma is not grown in the extracellular environment. Therefore, it is difficult to isolate high-purity DNA from phytoplasma. Nevertheless, six phytoplasma genomes have been completely sequenced, and twelve draft genomes have been prepared, which are also being completed (Kakizawa and Yoneda, 2015; Orlovskis, 2017). Determination of the complete genome sequence of phytoplasma provides the basis for the identification of virulence genes responsible for the development of changes in a plant and the causes of the productivity loss (Hogenhout and Martina, 2010). As these bacteria were not cultured *in vitro*, they were classified into 16Sr groups and subgroups based on the 16S ribosomal (16Sr) DNA sequence. Genomes of Onion Yellows M (OY-M) (Oshima, 2004) and Aster yellows witches'broom (AY-WB) (Bai et al. 2006) belonging, respectively, to 16SrIB and IA ribosom subgroups of '*Candidatus* (Ca) Phytoplasma asteris' species; *'Ca.* Phytoplasma australiense' (tuf-Australia subgroup; gr-A,) (Tran-Nguyen et al., 2008) PAa and SLY (strawberry lethal yellows) (Andersen et al., 2013); AT (apple proliferation) strains (Kube et al., 2008) of *'Ca.* Phytoplasma mali' were completely sequenced. Genomes of 'Candidatus Phytoplasma asteris' OY-V (onion yellows line V) (Kakizawa et al., 2014); '*Ca.*P. asteris' (Chrysanthemum yellows) (Pacifico et al., 2015); '*Ca.*P. asteris' WBD (Wheat

blue dwarf) (Chen et al. 2014); '*Ca.*P. solani' STOL (stolbur, 284/09 and 231/09) (Mitrovic et al., 2014); '*Ca.*P.aurantifolia' PnWB-16SrII (Peanut witches'broom) (Chung et al., 2013); '*Ca.*P. pruni' VAC (Vaccinium Witches'Broom) (Saccardo F. et al.2012); '*Ca.*P. pruni' ICP, MA1 (Italian Clover Phyllody phytoplasma MA) (Saccardo et al., 2012); '*Ca.*P. pruni' PoiBI, JR1 (Poinsettia branch-inducing phytoplasma JR1) (Saccardo et al., 2012); '*Ca.*P. pruni' MV1(milkweed yellows) (Saccardo et al., 2012) strains were read and draft variants were prepared (Figures 1 and 2).

In South America, a phytoplasma belonging to ribosomal subgroup 16SrIII-J has been reported in many crops. Here we report its genomic draft sequence, showing a total length of 687,253 bp and a GC content of 27.72%, 696 coding sequences, with 303 coding for hypothetical proteins, and 34 tRNAs, were identified and this is the first draft sequence of phytoplasma 16SrIII-J (Zamorano and Fiore, 2016).

Jujube 'Witches' Broom phytoplasma (JWB) is a kind of insect-transmitted and uncultivable bacterial plant pathogen causing a destructive Jujube disease. To understand its pathogenicity and ecology, the genome of a JWB phytoplasma isolate jwb-nky was sequenced and compared with other phytoplasmas enabled us to explore the mechanisms of genomic rearrangement. To improve the accuracy of the genome sequences, Illumina HiSeq 4000 platform and PacBio RS II platform (10 kb inserts library; Pacific Biosciences, Menlo Park, CA, USA) were used for the JWB-nky sequencing at the Beijing Genomics Institute (Wang et al., 2018).

The periwinkle leaf yellowing (PLY) disease was first reported in Taiwan in 2005. Comparative analysis with other available phytoplasma genomes indicated that this PLY phytoplasma belongs to the 16SrI-B subgroup in the genus, with "Candidatus Phytoplasma asteris" that causes the onion yellowing (OY) disease in Japan as the closest known relative (Shu-Ting et al., 2019).

MATERIALS AND METHODS

Genome analysis of uncultivable plant pathogenic phytoplasmas is hindered by the difficulty in obtaining sufficient quantities of phytoplasma enriched DNA. Similar approaches have been used for the isolation and sequencing of phytoplasma genomes (Patricia Carle et al., 2007). Phytoplasma DNA, rich in host plant DNA was purified by 4-fold bisbenzimide in CsCl density gradient (Kollar and Seemüller, 1989). Bisbenzimid Hoechst 33258 adenine + thymine (A + T) combines and their melting density is reduced. Thus, the phytoplasma DNA strip is characterized by a lower melting density compared to the strip of host plant DNA. Nucleic acids were isolated from different plants by CTAB- cetyltrimethylammonium bromide (Murray and Thompson, 1980) and purified nucleic acids were resolved in 1xTE buffer.

Phytoplasma DNA was isolated and cut into small fragments, then a whole genome shotgun library was created, or a faq library was subcloned into a plasmid vector and sequenced. However, despite the whole genome shotgun sequencing strategy allowed the completion of the four genome sequences, this strategy has some shortcomings: 1) Phytoplasma DNA is rich in AT. Especially when contaminated DNA of the host plant, less rich in AT, is present, it creates cloning issues. Host plant DNA phytoplasma is relatively more cloned, and the number of clones that hold host DNA is greater than those of phytoplasma DNA. This greatly increases the cost of sequencing, and it takes a long time to obtain pure phytoplasma DNA. 2) of the four phytoplasma genomes, at least three are rich in repeats. The plasmid rich in repeats easily recombines with E. coli, thus preventing cloning and sequencing of phytoplasma DNA sites which have no repeats. Next Generation Sequencing Technologies-454 Sequencing ™ (Roche Diagnostics Corporation), or Illumina sequencing technology (previous Solexia sequencing), opens new prospects for complete reading or mapping the phytoplasma genome. Contrary to other genomes, sequencing of the genome of SA213 (Quaglin et al., 2015) strain of the phytoplasma species *'Ca.* Phytoplasma phoenicium' related to the almond witches' broom disease was performed using Next Generation Sequencing Technologies (MiSeq Sequencing System, Illumina, San Diego CA, USA)

RESULTS AND DISCUSSION

Despite the fact that phytoplasmas are important and unique to agriculture, they remain the least characterized plant pathogens. Several hundred phytoplasma strains are known in the world today, and these strains are classified as 'Candidatus Phytoplasma' based on the variability of the 16SrRNA gene. Phytoplasma genome reading projects contributed to understanding the biology of phytoplasma. The data on nucleotide sequences of genomes of different phytoplasma species are compared in Table 1.

Based on the 16S ribosomal DNA (16Sr) sequence, phytoplasmas are divided into 3 various clusters (Hogenhout et al., 2008). The first cluster (Cluster I) includes Aster yellow (AY) 16SrI and stolbur (STOL) 16SrXII group phytoplasmas. These two groups have been diverged, but are more closely related to each other than the other phytoplasma groups. Three of the five completely sequenced phytoplasma genomes OY-M, AY-WB, and AUSGY belong to Cluster I. The fourth 'Ca. Phytoplasma mali 'phytoplasma belongs to Cluster II. One of the incompletely sequenced (draft) representatives of Cluster III, which includes most phytoplasmas, is SA213 strain of the phytoplasma species'Ca. Phytoplasma phoenicium' (Quaglin et al., 2015).

The sequenced phytoplasma genomes are classified into different subgroups, groups, and clusters, and useful information on the phylogenetics and diversity of phytoplasmas has been obtained. '*Ca*.Phytoplasma asteris' belongs to the OY-M AY-WB AY group and OY-M 16SrIB and16SrIA subgroups. Comparative analysis of the genomes of these two phytoplasmas allows understanding of how closely related phytoplasmas differ by the composition and structure of the genome. 'Ca.Phytoplasma austrliense' belongs to the STOL phytoplasma group and the new STOL (described as 'Ca.Phytoplasma solani') is an important pathogen for tomatoes, potatoes and grapes. 'Ca.Phytoplasma fragariae' is a pathogen of strawberries (Hogenhout et al., 2008).

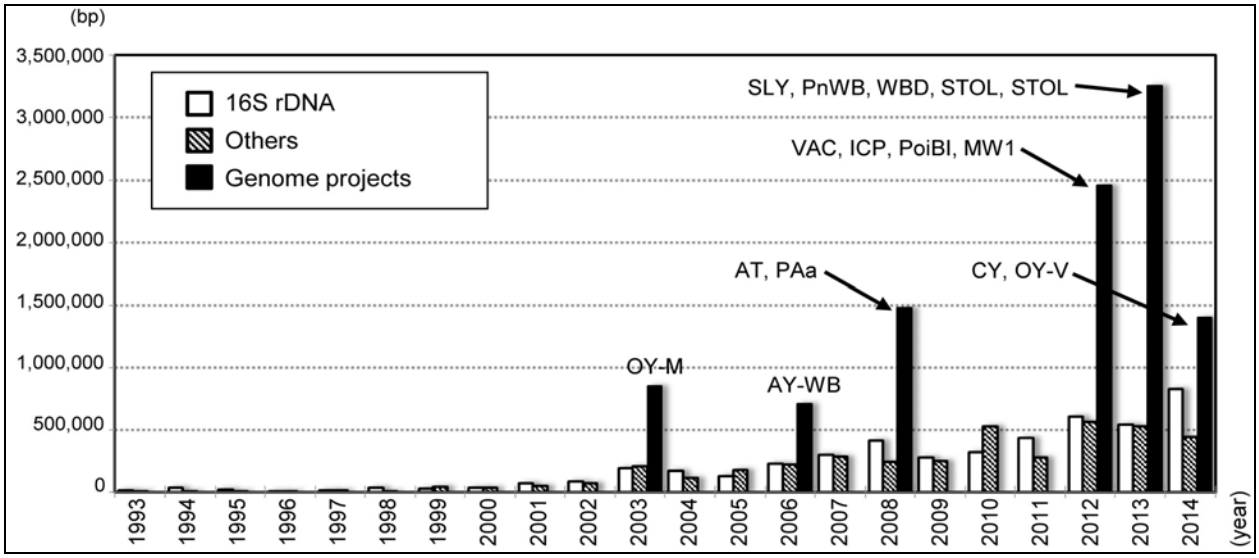
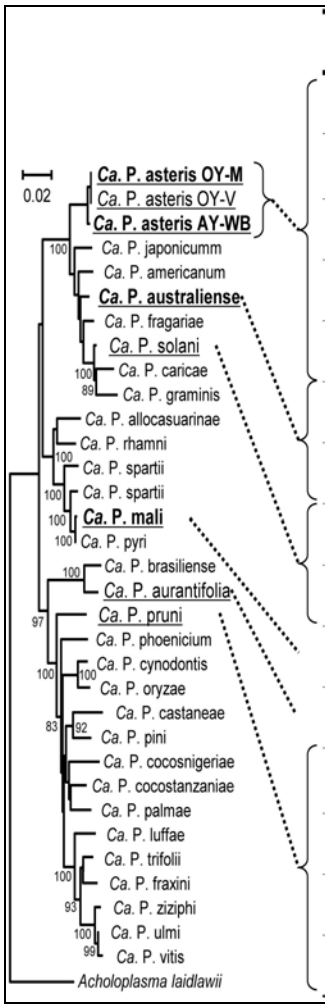


Fig. 1. Nucleotide length of phytoplasma 16S rDNA, genome projects and others. DNA sequences of phytoplasmas were retrieved from GenBank, separated into three categories (sequence containing 16S rDNA, obtained by genome sequencing projects, and others), and are shown by the released year. Sequences were collected by a criteria that phytoplasma or mycoplasma-like organism are included in the "organism" qualifier in each GenBank accession. Phytoplasma strain names of each genome project are shown. Abbreviations of these strains are shown in Figure 2. (Kakizawa and Yoneda, 2015).



Ca. species	strain	complete or draft	Length (bp)	% G+C	CDS	Acc. No.	reference
Ca. P. asteris	OY-M (onion yellows line M)	complete	860,631	28	754	NC_005303.2	Oshima *et al.*, 2004
Ca. P. asteris	OY-V (onion yellows line V)	draft	739,609	28	920	BBIY00000000.1	Kakizawa *et al.*, 2014
Ca. P. asteris	AY-WB (aster yellows-witches' broom)	complete	706,569	27	671	NC_007716.1	Bai *et al.*, 2006
Ca. P. asteris	CY (chrysanthemum yellows)	draft	659,699	28	711	JSWH00000000	(Pacifico *et al.*, 2015)*
Ca. P. asteris	WBD (wheat blue dwarf)	draft	611,462	27	562	AVAO01000000	Chen *et al.*, 2014
Ca. P. australiense	PAa (P. australiense Australian isolate)	complete	879,324	27	839	NC_010544.1	Tran-Nguyen *et al.*, 2008
Ca. P. australiense	SLY (strawberry lethal yellows)	complete	959,779	27	1126	NC_021236.1	Andersen et *al.*, 2013
Ca. P. solani	STOL (stolbur), strain 284/09	draft	570,238	29	520	NC_022588.1	Mitrović *et al.*, 2014
Ca. P. solani	STOL (stolbur), strain 231/09	draft	545,458	30	573	FO393428.1	Mitrović *et al.*, 2014
Ca. P. mali	AT (apple proliferation)	complete	601,943	21	544	NC_011047.1	Tran-Nguyen *et al.*, 2008
Ca. P. aurantifolia	PnWB (peanut witches' broom)	draft	562,473	24	450	AMWZ00000000	Chung et al., 2013
Ca. P. pruni	VAC (vaccinium witches' broom)	draft	647,754	27	677	AKIN00000000	Saccardo et al., 2012
Ca. P. pruni	ICP, MA1 (Italian clover phyllody, strain MA)	draft	597,245	27	650	AKIM00000000	Saccardo *et al.*, 2012
Ca. P. pruni	PoiBI, JR1 (poinsettia branch-inducing, JR1)	draft	631,440	27	654	AKIK00000000	Saccardo *et al.*, 2012
Ca. P. pruni	MW1 (milkweed yellows)	draft	583,806	28	565	AKIL00000000	Saccardo *et al.*, 2012

Fig. 2. Phytoplasma phylogenetic tree and summary of genome projects. A phylogenetic tree was constructed by the neighbourjoining method using 16S rDNA sequences from phytoplasmas and *Acholoplasma laidlawii*, and shown in the left. Phytoplasmas with complete or draft genomes are shown in bold plus underlined or underlined, respectively. *Ca*. P.: '*Candidatus* phytoplasma'. Summary of genome projects are shown in right. (Kakizawa and Yoneda, 2015).

The comparative analysis of the genomes of members of AY and STOL phytoplasmas provides insight into the similarities and differences in the composition and structure of phytoplasma genomes belonging to groups of Cluster I (Tran-Nguyen et al.,2008). Finally, 'Ca.Phytoplasma mali' belongs to the phytoplasma group Cluster II AY (16SrX), which also includes 'Ca.Phytoplasma pyri' (pear decline phytoplasma (PD) and 'Ca.Phytoplasma prunorum (Europian stone fruit yellows-ESFY).

The sequencing of the 'Ca.Phytoplasma mali' phytoplasma allows comparing the more closely related phytoplasmas of Cluster I and Cluster II (Kube et al.,2008). 'Ca.P. PAa and SLY (Tran-Nguyen et al., 2008; Andersen et al., 2013) isolates of the australiense 'phytoplasma species belong to the 16Sr XII-B group and differ greatly from other sequenced phytoplasma genomes, especially the SLY strain, in the number of genes and the size of the genome.

Extensive information on the functions of genes encoded in completely sequenced genomes of phytoplasmas, repetitive sequences in the genome, DNA-natural plasmids out of their chromosome, comparative results of the genome analysis, and candidate virulence factors of the phytoplasma was obtained.

Table 1. The main properties of complete genomes sequences of phytoplasma and *A.laidlawii*

***'Ca.* Phytoplasma' species**	**'Ca.P.asteris'**			**'Ca.P. australiense'**		**'Ca.P.a mali '**	**'Ca. P. ziziphi'**	**A.laidlawii PG-8A**
Ştamlar	**OY-M**	**AY-WB**	**PLY**	**Rp-A**	**SLY**	**AT**	**JWB**	**A. *laidlawii***
16S rDNA groups	**IB**	**IA**	**IB**	**XIIB**		**X**	**VB**	
Klasters	I	I		I	I	II		
Chromosom size(bp.)	853,092	706,569	824,596	879,959	959,779	601,943	750,803	1 496,992
Chromosom composition	Circular	Circular	Circular	Circular	Circular	Linear	Circular	Circular
Quantity of G+C (%)	27,76	26,89	27.6	27,42	27	21,39	23.3	31,93
Quantity of G+C (%) of protein-coding genes	29,09	28,54		28,72		22,58	----	32,23
Protein-coding regions (%)	73,1	73,7	70.6	64,1	78	76,3		90,7
Coding sequences	793	708		839		536		
Protein coding genes (pseudogenes)	752	776	775 (62)	684(155)		481(16)	694	1380(11)
Protein-encoding genes with determined functions	446	450		502	528	338		
(Conservation) hypothetical proteins[a]	308	221	264	270	249	159		
Single copy proteins	486	482		482		408		
Multi copy proteins	268	191		202		89		
Multi copy proteins PMUs (potencial mobil units)	175	134		143		4		
Tra5[b] transpozaza-like	7(6)	6*(20)		5(1)		(1)		
Fragmented genes	46	102		159		16		
Average size of ORF (bp)	829	776		839	1126	955		984
Protein coding genes/kb	0.881	0.949		0.777		0.799		0.921
No. of tRNA genes	32	31	32	35	35	32	31	34
No. of rRNA operons	2	2	6	2	2	2	2	2
Extrachromosomal DNA-plasmid-like elements	2	4	0	1	0	0		----
Access number	AP006628.2	CP000061.1	SRMC01000001 SRMC01000008	AM422018.1	NC_021236.1	CU469464.1		CP000896.1

The information were received Oshima et al. (2004), Tran-Nguyen et al. (2008) and Kube et al. (2008).
[a]Annotations of protein-coding genes - "hypothetical proteins" and "conservative hypothetical proteins" were selected and numbered from GenBank by accession numbers NC_005303, NC_007716, NC_010544, and NC_011047, respectively OY-M, AY-WB, 'Ca.Phytoplasma australiense' and 'Ca.Phytoplasma mali' are included in the complete chromosome sequence.
[b] Full-length protein sequence of tra5 gene of the AY-WB (GenBank accessionYP-456371.1) was extracted from GenBank with using blastp against protein sequences NC_005303, NC_007716, NC_010544, and NC_011047. The full length of transposases and cut transposes was analyzed in the file output. (the last is indicated by brackets).
***** Two of the six tra5 genes contain two ORFs that in the case of frame sliding may be to make one transposition.

Phytoplasmas are absolute symbionts of plants and insects, causing various cultures to lose significantly their productivity. Complete and incomplete sequencing of the genomes revealed repetitive genes of approximately 20 bp in many phytoplasmas. These "potential mobile units" (PMUs) look like the composition of replicative transposons. PMUs preserve several genes for recombination, some of which are supposedly virulence genes. As a result of genome equalization, PMUs are involved in the instability and recombination of genomes in the phytoplasma. Hogenhout and co-workers reported that the Witches' Broom (AY-WB) may be linear in the aster yellows strain of phytoplasma, on the PMU chromosome and circular out of the chromosome. The number of copies of the circular forms is higher in the insect vector than in plants, and the expression levels of genes in the PMU are also higher in insects. These observations suggest that PMUs can participate not only in mobile elements but also in phase-

shifting mechanisms that allow phytoplasma to adapt to different hosts (Matt Dickinson, 2010).

JWB phytoplasma now classified as 'Ca. P. ziziphi', belongs to elm yellows group (16SrV) subgroup B based on analyses of 16SrDNA sequences (Jung HY et al., 2003). Based on PHIbaes analysis, a large number of genes were genome-specific and approximately 13% of JWB phytoplasma genes were predicted to be associated with virulence. Although transporters for maltose, dipeptides/oligopeptides, spermidine/ putrescine, cobalt, Mn/Zn and methionine were identified, KEGG pathway analysis revealed the reduced metabolic capabilities of JWB phytoplasma. Comparative genome analyses between JWB phytoplasma and other phytoplasmas shows the occurrence of large-scale gene rearrangements. Comparative genomic analysis revealed that, although jwb-nky is closely related to 'Ca. P. pruni' according to phylogenetic analysis, the gene syntety between the two phytoplasmas is low (Wang et al., 2018).

The de novo genome assembly of PLY phytoplasma produced eight contigs with a total length of 824,596 bp. The annotation contains 775 protein coding genes, 63 pseudogenes, 32 tRNA genes, and two sets of rRNA operons. To characterize the genomic diversity across populations, a second strain that infects green onions in Yilan was collected for re-sequencing analysis. Comparison between these two strains identified 337 sequence polymorphisms and 10 structural variations. The metabolic pathway analysis indicated that the PLY phytoplasma genome contains two regions with highly conserved gene composition for carbohydrate metabolism. Intriguingly, each region contains several pseudogenes and the remaining functional genes in these two regions complement each other, suggesting a case of duplication followed by differential gene losses (Shu-Ting et al., 2019).

The results from this work provided insights into the genomic diversity among phytoplasmas at the levels of populations and species. When comparing across populations, differentiations in plant hosts may play a more important role than geographical distance in shaping genetic diversification. However, such inference requires more comprehensive sampling of closely related phytoplasma genomes for further tests. At the level of species or sub-species comparisons, mobile genetic elements such as PMUs played an important role in genome rearrangement, gene content evolution, and horizontal gene transfer. Particularly, the exchange of effector genes among different phytoplasma lineages may facilitate their adaptation. Furthermore, duplication of chromosomal segments followed by differential gene loss, such as that commonly observed after whole genome duplication in eukaryotes could be another evolutionary process the drives the genome divergence of these bacteria. This finding provided novel insights into the genome evolution of pathogenic bacteria with highly reduced genomes.

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'*Ca.* Phytoplasma' Növlərinin Genom Ardıcıllıqlarının Müqayisəli Analizi

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Bakteriyanın «*Candidatus* Phytoplasma» cinsi kultivasiya olunmayan, floema şirəsi ilə qidalanan həşəratlarla ötürülən hüceyrədaxili bitki patogenləridir. Onların böyük olmayan genomlarında əsas metabolitlərin genləri yoxdur və həmin metabolitləri sahib bitki və həşəratlardan mənimsəyirlər. Altı fitoplazma ştamının genomlarının tam sekvensi nəticəsində bitki və həşəratlar ilə fitoplazmanın qarşılıqlı təsirinin molekulyar səviyyədə aydınlaşdırılmasında böyük uğurlar əldə olunmuşdur. Sekvens olunmuş altı fitoplazma genomunun müqayisəli analizi genomun ölçüsündə, tərkibində, metabolik yollarda və təkrarların sayında müxtəlifliyin olduğunu aşkar etmişdir. Hesab olunur ki, fitoplazmaların genomlarının sürətli təkamülə məruz qalmaları onların həyat tsiklinin nəticəsi ola bilər. Fitoplazmaların bitkilər və həşəratlar arasında və təbiətdə fasiləsiz yaşama və yayılma tsikli üçün hər iki orqanizm tələb olunur. Bu ətraf mühitin geniş bir diapazonuna, onların sahib bitkilərinin floemasına, həşərat sahiblərinin bağırsaq lümeninə, hemolimfa, tüpürcək və müxtəlif orqanlarda hüceyrədaxili nişalara adaptasiyasını tələb edir. Fitoplazma genomunun oxunması potensial virulent zülalların sayının identifikasiyasına gətirib çıxarmışdır ki,onlardan bəziləri funksional baxımdan xarakterizə olunmuş və həşaratların bitkilərə daxil olmasında rolları sübut edilmişdir. Bunlar həşəratın bağırsaq mikroifilamentlərinin və bitkinin hüceyrə nüvəsi hədəfi olan sekresiya zülallarının birləşməsinə cəlb olunmuş fitoplazma hüceyrəsinin səthində yerləşən zülalladır. Əksər fitoplazma genomlarında böyük təkrarlar vardır ki, bu da fitoplazmanın virulentliyində təzahür edir.

Açar sözlər: *Fitoplazma DNT-si, genom ardıcıllığının tam və qaralama variantları, potensial mobil vahidlər*